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The Mdm2 Ubiquitin Ligase Enhances Transcriptional Activity of Human Papillomavirus E2▽

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The regulation of human papillomavirus (HPV) gene expression by the E2 protein is a critical feature of the viral life cycle. Previous studies have shown an important role in transcription for the ubiquitin-proteasome pathway, but its role in HPV gene expression has not been addressed. We now show that HPV E2 requires an active proteasome for its optimal transcriptional activator function. This involves an interaction with the Mdm2 ubiquitin ligase, which together with E2 acts synergistically to activate the HPV type 16 promoter. We also show that HPV E2 recruits Mdm2 onto HPV promoter sequences, providing an explanation for this cooperative activity.

Viruses are intracellular parasites that rely upon their host cells for the completion of their productive life cycles. Therefore, viruses need to use cellular machinery to support their life cycles. The replication of the double-stranded DNA genomes of human papillomaviruses (HPVs) is directly regulated by the viral helicase, E1, and the transcriptional regulator, E2 (19). The E2 protein is a multifunctional, DNA binding protein, consisting of a transactivation domain in the N-terminal half of the protein, a middle nonconserved hinge region, and a C-terminal domain that mediates DNA binding and protein dimerization. Four E2 binding sites are present in the long control regions (LCRs) of HPV genomes (30): two sites flank the viral origin of replication, a third site lies directly upstream of the early promoter which controls expression of viral early genes (24, 27), and a fourth site lies at the 5′ end of the LCR. The binding of E2 to the LCR facilitates the binding of the E1 helicase to the viral origin of replication for the initiation of DNA replication and the binding of transcription factors, such as the TATA-binding protein and Sp1, that are required for viral gene expression (29). In addition, a number of direct interactions of E2 with cellular proteins such as Brd-4 (18), TopBP1 (5), and Brm (11) further contribute to the regulation of viral gene expression.

The role of the ubiquitin-proteasome machinery in transcriptional regulation is now well recognized (20), although how it functions most likely varies depending upon the precise promoter complex. It may involve a form of licensing where ubiquitination links the activities of specific transcription factors to their own destruction (26). Alternatively, ubiquitin-modified transcription factors may recruit protein-remodeling factors to the promoter and enhance corepressor/coactivator exchange (7, 21). Specific examples of recruitment and enhancement include the regulation of gene expression by c-myc and steroid hormone receptors (1, 23) as well as the activation of the hTERT promoter by HPV E6, which is dependent on the interaction between E6 and the E6AP ubiquitin ligase (15).

In the case of the HPV promoter and, more specifically, of the function of E2, the involvement of the proteasome in transcriptional regulation has not been documented. In order to investigate this, we first analyzed the effects of proteasome inhibition upon E2 transcriptional activity (8). To do this, U2OS cells were transfected with an E2-responsive luciferase reporter construct (p6xE2BS-Luc/E2-Luc reporter plasmid, kindly provided by Ian Morgan) together with an untagged HPV type 16 (HPV-16) E2 expression plasmid (22). After 24 h, the cells were treated in the presence of 50 μM of the proteasome inhibitor CBZ (Sigma) or dimethyl sulfoxide, as a control, for a further 5 h. Then, the cells were lysed, and their luciferase activity was determined using the Dual-Luciferase assay kit (Promega). The results obtained are shown in Fig. 1a, left panel, and demonstrate a clear inhibition of E2 transcriptional activity following proteasome inhibition. To investigate the specificity of proteasome inhibition on E2 transcriptional activity, we also included a glucocorticoid receptor (GR)-responsive plasmid, MMTV-Luc (kindly provided by Olivier Kassel [10]), which was shown previously to be unaffected by proteasome inhibition (14). Figure 1a, right panel, shows that CBZ did not suppress dexamethasone (Dex)-induced GR transactivation. In the case of other transactivators, such as p53 and E2F, proteasome inhibition did affect their transcriptional activity (data not shown), which is in agreement with previously published data (13, 31).

Treatment with proteasome inhibitors is known to provide a block to the entire proteasome machinery, but this result led us to speculate whether or not a known ubiquitin ligase might be involved in E2 transcriptional activity. We analyzed the effects of a number of ubiquitin ligases that had previously been linked to HPV function on E2 transcriptional activity. Therefore, plasmids expressing E6/AP (kindly provided by Martin Scheffner), Mdm2 (kindly provided by Claudio Schneider), β-TrCP (16), Smurf, and Cbl were cotransfected with E2 and E2-Luc expression constructs, and luciferase assays were performed after 24 h. The results obtained are shown in Fig. 1b, where it can be seen that Mdm2 induces a dramatic increase in the transcriptional activity of E2. In contrast, none of the other

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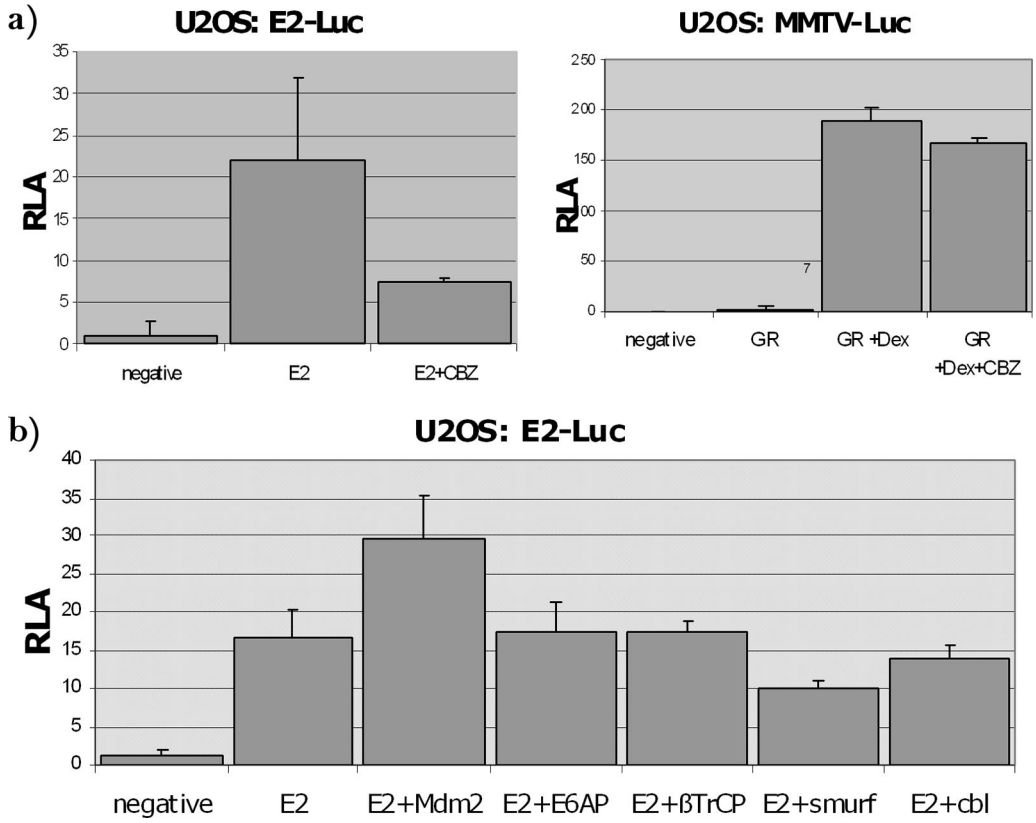

FIG. 1. The role of the proteasome machinery in the transcriptional activity of E2. To assess E2 transcriptional activity, luciferase assays were conducted in U2OS cells transfected with a reporter construct containing E2 binding sites upstream of the luciferase gene (E2-Luc), plus the *Renilla* luciferase gene as a transfection control and an untagged HPV-16 E2 expression plasmid. Representative results of three experiments are shown together with standard deviations. RLA, relative luciferase activity. (a, left) E2 transcriptional activity in the presence or absence of proteasome inhibitors (CBZ). (a, right) The effects of CBZ on Dex-induced GR transactivation. U2OS cells were transfected with GR and MMTV-Luc plasmids and treated with 10 nM Dex (Sigma) for 16 h, followed by treatment with CBZ for a further 5 h. (b) The effects of various ubiquitin ligases on E2 transcriptional activity. Cells were transfected with the reporter plasmids and E2 in the presence of expression plasmids of the indicated ubiquitin ligases.

ubiquitin ligases had any significant effect on the ability of E2 to activate transcription, highlighting the specificity of the Mdm2 activity with respect to E2. These results suggest that the Mdm2 ubiquitin ligase activity might mediate this effect.

The C-terminal end of Mdm2 contains a RING domain conferring E3 ligase activity which mediates its ability to target several proteins for degradation, including p53 (2), pRb (28), and the androgen receptor (9). In addition, the middle region of Mdm2 also contains nuclear export and nuclear localization sequences and was shown to increase the transcriptional activity of a number of factors such as the p53 homolog p63 (3) and estrogen receptor α (25). To further investigate the role of Mdm2 in the transcriptional activity of E2, we repeated the luciferase assay using a point mutant of Mdm2 that no longer has ubiquitin ligase enzymatic activity (Mdm2C462A; kindly provided by Claudio Schneider) and including proteasome inhibition as an additional control. Figure 2a shows again that Mdm2 exerts a potent stimulatory effect upon E2 transcriptional activity and that this is abolished following treatment with CBZ. Most importantly, the Mdm2C462A mutant is also defective in its ability to stimulate E2 transcriptional activity, demonstrating that Mdm2 ubiquitin ligase activity is required for its ability to upregulate the E2 transcriptional activator function.

To exclude a possible indirect role for p53 in these assays, the same experiment was repeated with SAOS-2 cells ($p53^{-/-}$ $pRb^{-/-}$) and H1299 cells ($p53^{-/-}$). Figure 2b shows that Mdm2 cooperates with the transcriptional activation function of E2 in both cell lines, demonstrating that this is independent of Mdm2's ability to degrade either p53 or pRb. Furthermore, expression of Mdm2 in increasing amounts in H1299 cells resulted in a progressive increase in the transcriptional activity of E2 (Fig. 2b, right), demonstrating that Mdm2 cooperates with E2's transcriptional transactivation function in a dose-dependent manner. We also tested whether inhibiting the activity of endogenous Mdm2 would affect the transcriptional activity of E2. To do this, SAOS-2 cells transfected with E2 and E2-Luc plasmids were treated with two different Mdm2 inhibitors, Nutlin-3 and Mdm2 E3 ligase inhibitor, which were used previously to inhibit Mdm2 functions that are independent of p53 degradation (4). The results obtained are shown in Fig. 2c, where it can be seen that inhibiting endogenous Mdm2 activity by either inhibitor results in a decrease in E2's transcriptional activity. Finally, we also wished to determine whether or not

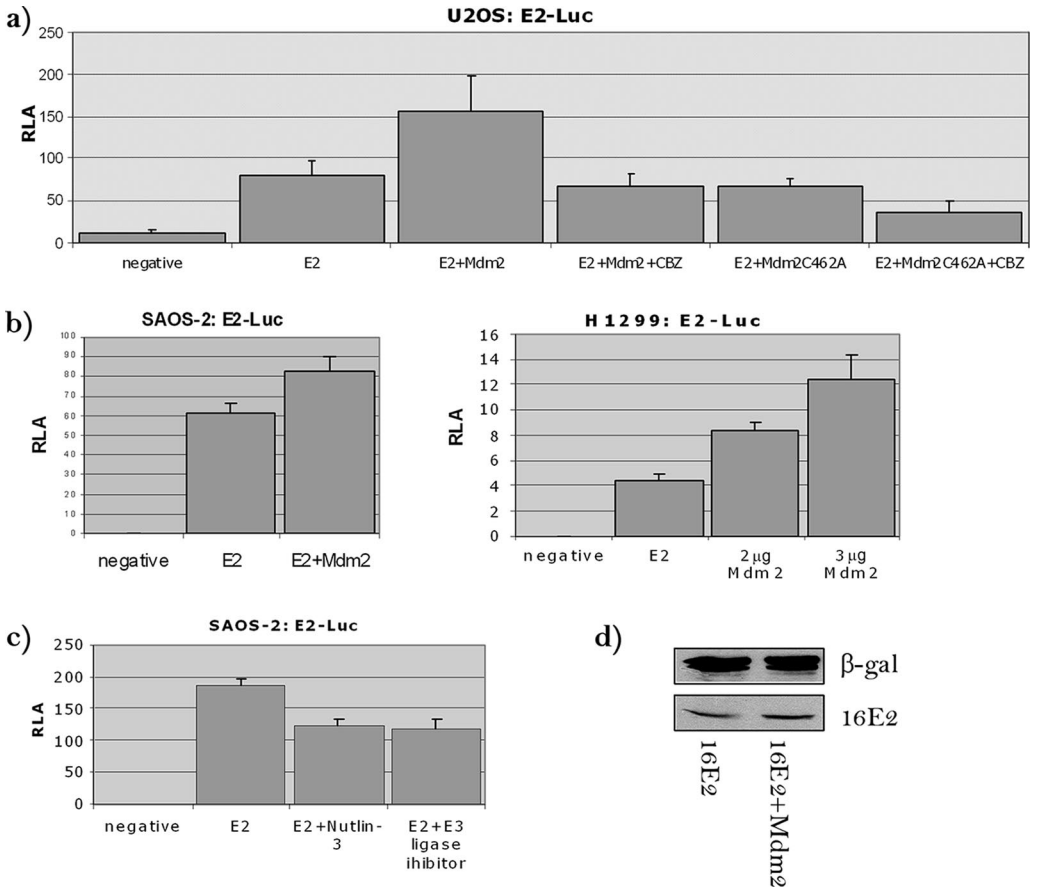

FIG. 2. Mdm2 enhances the transcriptional activity of E2. (a) The ubiquitin ligase activity of Mdm2 is required to enhance E2 transcriptional activity. A luciferase assay was conducted in the presence of Mdm2C462A (ligase-dead mutant of Mdm2) and CBZ. RLA, relative luciferase activity. (b) Mdm2 enhances E2-mediated transcriptional activity in SAOS-2 ($p53^{-/-}$ $pRb^{-/-}$) (left panel) and H1299 ($p53^{-/-}$ $pRb^{+/+}$) (right panel) cells. Cells were transfected with the reporter construct and an E2 expression vector with or without the expression of Mdm2 as shown in panel a. (c) The transcriptional activity of E2 is reduced in the presence of Mdm2 inhibitors. SAOS-2 cells were transfected as shown in panel b. Five hours prior to harvest, cells were treated with Nutlin-3 (10 μM; Sigma) or Mdm2 E3 ligase inhibitor (10 μM; Calbiochem). (d) The effect of Mdm2 expression on E2 levels was tested by expressing both proteins in U2OS cells. At 24 h after transfection, protein levels were analyzed by Western blotting using antibodies against 16E2 or β-Gal as a transfection control.

Mdm2 can affect the levels of E2 protein expression. To do this, U2OS cells were transfected with the E2 expression plasmid together with Mdm2 and a β-galactosidase (β-Gal) marker for transfection efficiency, and 24 h later, the levels of E2 expression were ascertained by Western blot analysis using polyclonal anti-E2 antibodies (17). The results in Fig. 2d show that the levels of E2 remain largely unchanged with respect to the β-Gal loading control in the presence of Mdm2. This demonstrates that the increased transcriptional activation exhibited by E2 in the presence of Mdm2 is not the result of increased levels of E2 expression. Taken together, these results demonstrate that E2 requires the functional proteasome machinery for optimal transcriptional activity and that this appears to involve the Mdm2 ubiquitin ligase.

Since we had seen that Mdm2 affects E2 transcriptional activity without affecting its protein expression levels, we then sought to examine whether Mdm2 and E2 can interact. Using an in vitro binding assay, bacterially expressed glutathione *S*-transferase (GST) fusion proteins (6) were incubated with in vitro translated and radiolabeled Mdm2 (as described in reference 8). Figure 3a shows that Mdm2 can bind to GST-16E2, albeit less strongly than it binds to GST-p53; no binding is seen with GST alone. To identify the region of E2 that mediates its interaction with Mdm2, we used a number of GST-tagged E2 fragments, including the N- and C-terminal domains and truncation fragments of the C-terminal domain (Fig. 3b, right). These were previously described in reference 6. Figure 3b shows that E2 binds to Mdm2 mainly through its C-terminal half and, more specifically, through a region spanning amino acid residues 322 to 335.

In order to verify that E2 and Mdm2 can form a complex in vivo, we performed a coimmunoprecipitation assay with 293 cells that were transfected with either Mdm2 alone or Mdm2 plus GFP-tagged E2. At 24 h posttransfection, E2 was immunoprecipitated from cell extracts, using antibodies against green fluorescent protein (GFP) (polyclonal; Santa Cruz), for 3 to 4 h. After extensive washing, the immunoprecipitate was analyzed by Western blotting, using an anti-Mdm2 monoclonal antibody (kindly provided by Giannino Del Sal). Figure 3c shows that a complex between Mdm2 and E2 can be detected

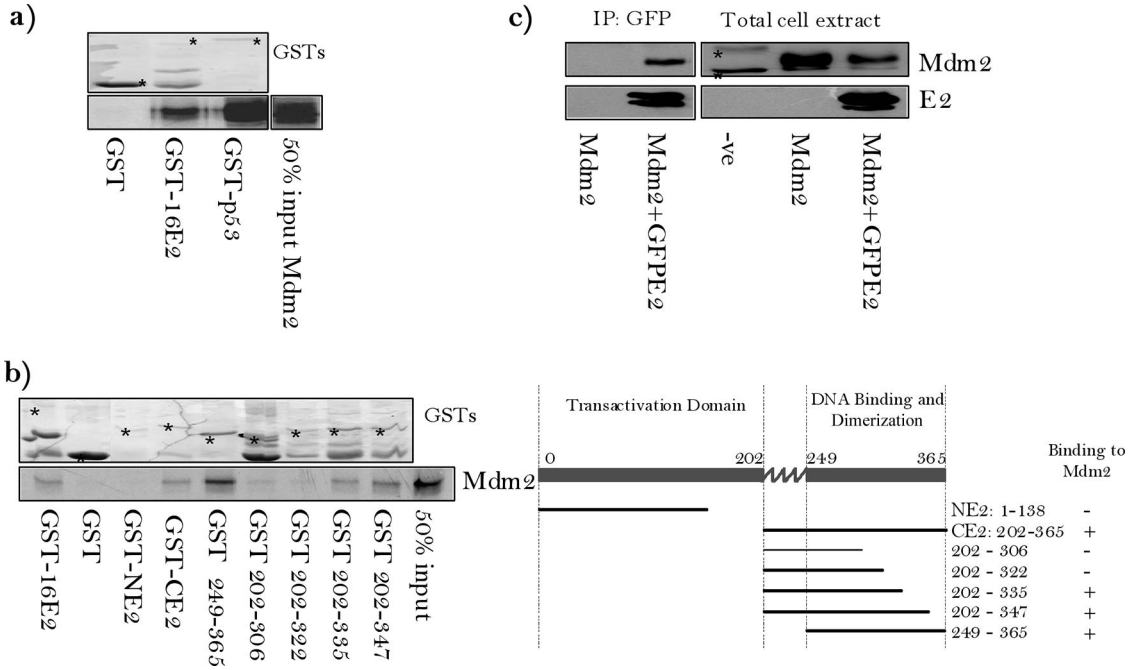

FIG. 3. Mdm2 interacts with 16E2 in vitro and in vivo. (a) In vitro-translated and radiolabeled Mdm2 was incubated with bacterially purified GST-tagged E2. GST alone and GST-p53 were included as negative and positive controls, respectively. Bound proteins were analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. The Coomassie blue stains of the GST inputs are also included in the top panel. An asterisk indicates the full-length GST fusion proteins. (b) Mdm2 binds to the C-terminal region of E2. GST-16E2 and a number of GST-tagged fragments of E2 (right) were incubated with in vitro-translated and radiolabeled Mdm2, and bound proteins were analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. Fifty percent of the input is included, and the GST inputs are shown in the bottom panel and are stained with Coomassie blue. An asterisk indicates the full-length GSTs. NE2, N-terminal half of E2 protein; CE2, C-terminal half of E2 protein. (c) E2 and Mdm2 bind in vivo. 293 cells were transfected using either Mdm2 alone or Mdm2 with GFP-tagged E2. Cell extracts were immunoprecipitated (IP) using polyclonal anti-GFP antibodies, followed by Western blot analysis using antibodies against Mdm2 or GFP. An asterisk indicates nonspecific bands. -ve, untransfected cells.

in cells expressing Mdm2 and E2 but not in cells expressing Mdm2 alone, demonstrating that E2 and Mdm2 can form a complex in vivo.

Since the recruitment of ubiquitin ligases to promoters is one means by which the proteasome can be linked directly to transcriptional activation, we examined whether E2 could similarly recruit Mdm2 to the HPV promoter by using a chromatin immunoprecipitation assay (Upstate Biotechnology). 293 cells were transfected with the E2-Luc construct together with GFP-E2 and FLAG-Mdm2 expression constructs (kindly provided by Ian Morgan and Giannino Del Sal, respectively). At 24 h posttransfection, cells were cross-linked with 1% formaldehyde for 10 min at 37°C, lysed, and sonicated to generate 200- to 1,000-bp DNA fragments. Cell lysates were then incubated with the indicated antibodies, and immunoprecipitated complexes were analyzed by PCR to detect protein interaction with DNA. Initially, we verified that GFP-E2 and FLAG-Mdm2 could form a complex in vivo by using anti-FLAG antibodies (M2; Sigma) to immunoprecipitate Mdm2, followed by Western blot analysis using anti-E2 antibodies (17). The results in Fig. 4a again confirm the binding of both proteins in vivo. In parallel, cell extracts were immunoprecipitated using anti-GFP (Clontech), anti-FLAG (Sigma), or anti-HA (Roche) antibodies, and the coprecipitated DNA was analyzed by PCR using primers complementary to sequences present in the luciferase gene (12). The results in Fig. 4b show that, as expected, GFP-E2 binds strongly to DNA that contains E2 binding sites (lane 5). Interestingly, by using anti-FLAG antibodies, we see that Mdm2 coimmunoprecipitates with DNA in the presence of E2 (Fig. 4b, lane 6); in contrast, there is no coimmunoprecipitation of DNA in the absence of E2 (Fig. 4b, lanes 1 through 3) nor when using anti-HA antibodies as a control (Fig. 4b, lane 4). These results strongly suggest that Mdm2 can bind to E2 when it is bound to its cognate DNA recognition sequences.

This study shows for the first time a role for a ubiquitin ligase, Mdm2, in the transcriptional activity of HPV-16 E2. This observation is supported by the abilities of Mdm2 and E2 to interact in vivo and in vitro. The effects of Mdm2 on E2 transcriptional activity are not due to alterations in the protein levels of E2 but most probably due to the ability of E2 to recruit Mdm2 to the HPV promoter. The exact molecular mechanisms by which Mdm2 influences the transcriptional activity of E2 are still to be identified. Mdm2 may increase the ubiquitination of E2, it might modulate the recruitment of cellular factors that may facilitate E2 transcriptional activity, or it may direct the degradation of some of the transcriptional machinery, thereby allowing increased transcriptional licensing and processivity. At present we cannot completely differentiate between these different possibilities; however, a role in transcriptional licensing seems to be most likely. Clearly, an active proteasome is required, which

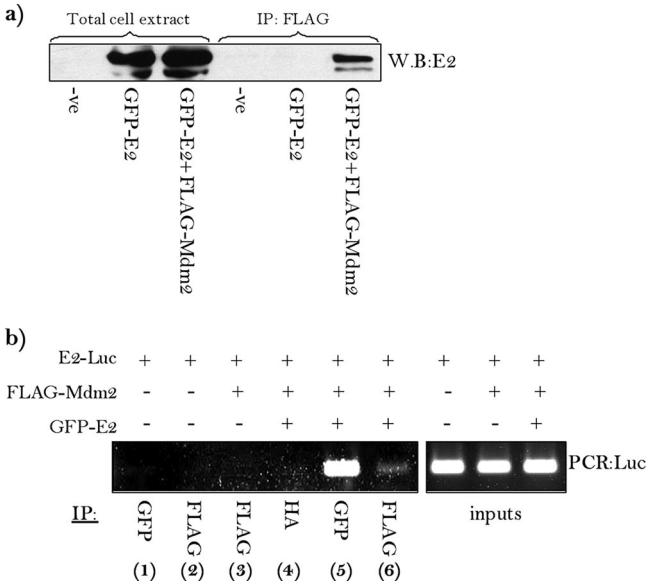

FIG. 4. Mdm2 binds to E2 on DNA. (a) FLAG-tagged Mdm2 immunoprecipitates with GFP-tagged E2. 293 cell extracts expressing GFP-E2 with or without the coexpression of FLAG-Mdm2 were immunoprecipitated (IP) using anti-FLAG antibodies, and immunoprecipitated proteins were detected by Western blotting (W.B) using antibodies against E2. -ve, untransfected cells. (b) Mdm2 immunoprecipitates with DNA-bound E2. 293 cells were transfected with a E2-Luc construct along with different combinations of FLAG-Mdm2 and GFP-E2 expression plasmids, and cell extracts were immunoprecipitated using the indicated antibodies. The coprecipitated DNA was analyzed by PCR using primers that are complementary to the luciferase gene.

tends to rule out the simple ubiquitin modification of E2 and suggests rather that the proteolytic machinery is involved. In addition, a mutant Mdm2 lacking ubiquitin ligase activity is unable to increase E2 transcriptional activation, and two small molecule inhibitors of Mdm2 also reduce E2 transcriptional activity, thereby demonstrating that the ubiquitin ligase activity of Mdm2 is also important. Finally, a clear complex between E2 and Mdm2 is seen when E2 is bound to its cognate DNA recognition sequence, demonstrating that E2 can actively recruit Mdm2 to the HPV promoter. Combining these three critical features supports a role for Mdm2 in the transcriptional licensing of HPV-16 E2-activated gene expression. Future studies will now aim to identify the substrates of this E2/Mdm2/DNA complex.

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